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Chemical Nature and Biological Effects of the Aflatoxins[1]

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INTRODUCTION

The aflatoxins represent a group of secondary fungal metabolites which were discovered as contaminants of certain lots of animal feeds. These compounds have a high order of acute toxicity to many animal species, and have been shown to possess potent carcinogenic properties in several animal species. Their discovery in agricultural commodities and the subsequent demonstrations of various biological effects resulting from ingestion of contaminated diets by animals have emphasized the potential public health hazards which might arise from contamination of the food supply by so-called "mycotoxins" (38). These factors have recently stimulated considerable research activity dealing with many aspects of the aflatoxins and other toxic mold metabolites.

Toxicity syndromes of domestic animals ultimately attributed to aflatoxins were first recognized in England in 1960 and 1961. A detailed account of the early outbreaks of unexplained mortality has been given elsewhere (3, 38), and pertinent information need only be briefly summarized here. The first report of the syndrome in young turkeys (14) was followed within a short time by similar incidents in ducklings and chickens (11), as well as in swine (32, 43) and calves (44). The common factor in these episodes was a shipment of Brazilian peanut meal used in the animal rations. It was found that the active principle could be extracted from toxic meals (4, 55), and such extracts reproduced the toxicity in susceptible species. Subsequently, many samples of peanut meals were tested for toxicity, with the result that some samples from at least 13 producing countries were found to be contaminated with the toxic agent (3). It has since been suggested by examination of many types of agricultural commodities that peanut meals are probably more frequently contaminated than other crops (34). This has been attributed to such factors as high moisture content or methods of harvest and storage which tend to favor growth of contaminating molds. The discovery of a highly contaminated cottonseed meal (42) illustrates the lack of a specific substrate-aflatoxin relationship.

Sargeant et al. (54, 56) associated toxicity with heavy mold infestation of feedstuffs and demonstrated that the toxic compounds were produced by certain strains of *Aspergillus flavus* isolated from such meals. The generic name "aflatoxins" was subsequently applied to the group of toxic compounds produced by this fungus. A recent report (35) indciates that they may also be elaborated by certain isolates of *Penicillium puberulum*.

CHEMICAL NATURE OF AFLATOXINS

Isolation

Isolation of the aflatoxins from toxic meals was greatly facilitated by the discovery that they were strongly fluorescent in ultraviolet light. This

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property has also provided a convenient means for monitoring of isolation and purification procedures. The original investigations (4, 55) demonstrated that the compounds were extractable with methanol. A variety of extraction procedures have since been developed for use with various natural products or mold cultures on natural substrates, particularly in connection with chemical assays of agricultural commodities for aflatoxin contamination. These include, for example, aqueous methanol (21, 46, 64), aqueous acetone (52), and a hexane-acetone-water azeotrope (30). These solvents appear to be efficient in extracting the compounds, particularly when they are present in small concentration.

In the production and isolation of quantities of aflatoxins from mold cultures on solid substrates, a convenient extraction and concentration procedure involves total extraction of the culture with chloroform and subsequent precipitation of the aflatoxins in petroleum ether (7, 8). The aflatoxins produced in cultures on liquid media are almost quantitatively removed by partitioning into chloroform (1).

Extracts produced by these procedures usually contain complex mixtures of fluorescent compounds, which are separable into their individual components by chromatographic techniques. Resolution on filter paper is incomplete (56), but is greatly improved by the application of thin-layer chromatographic procedures. Although several such systems have been developed, including the use of alumina as the support medium (15), the conditions most widely used involve separation on silica gel plates developed with 3 to 5% methanol in chloroform (8, 26, 46). Further improvement in resolution has been reported in a partitioning system comprising kieselguhr plates developed with formamide-benzene-water (1).

When such chromatograms of extracts containing aflatoxins are viewed under ultraviolet light, a complex array of fluorescent compounds is generally present. The known aflatoxins comprise four of the components. Two emit blue visible light, and were therefore named aflatoxins B_1 and B_2; two fluoresce yellow-green (aflatoxins G_1 and G_2). On silica gel plates developed in (97:3) chloroform-methanol (8), aflatoxin B_1 migrates with an R_F in the order of 0.56; B_2, 0.53; G_1, 0.48; and G_2, 0.46, although absolute R_F values are poorly reproducible. The amounts and relative proportions of these four compounds present in culture extracts are variable, depending on such factors as mold strain, medium composition, and culture conditions. Typically, aflatoxins B_2 and G_2 are present in small relative amounts, whereas B_1 is usually present in largest yield.

TABLE 1. *Summary of physical data on aflatoxins*

Aflatoxin	Molecular formula	Molecular weight	Melting point	$[\alpha]D^{23}$
			C	
B_1	$C_{17}H_{12}O_6$	312	268–269*	−559
B_2	$C_{17}H_{14}O_6$	314	286–289*	−492
G_1	$C_{17}H_{12}O_7$	328	244–246*	−533
G_2	$C_{17}H_{14}O_7$	330	237–240*	−473

* Decomposes.

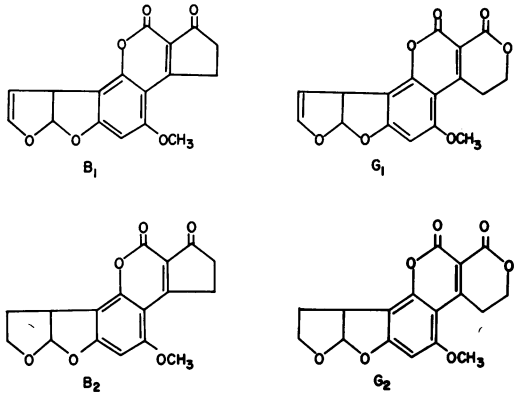

FIG. 1. *Structures of the aflatoxins.*

Physical Properties and Structures

These four compounds were originally isolated by groups of investigators in England (45, 56) and Holland (66). The molecular formula of aflatoxin B_1 was established as $C_{17}H_{12}O_6$, and of aflatoxin G_1 as $C_{17}H_{12}O_7$; aflatoxins B_2 and G_2 were found to be the dihydro derivatives of the parent compounds, $C_{17}H_{14}O_6$ and $C_{17}H_{14}O_7$ (33). Some physical properties of the compounds are summarized in Table 1.

Structures based largely on interpretation of spectral data were proposed for aflatoxins B_1 and G_1 in 1963 (7, 8), and for B_2 (23, 67) and G_2 shortly thereafter. These are shown in Fig. 1. The proposed structure of G_1 has been supported by X-ray crystallography (24). Laboratory synthesis of the compounds has not yet been accomplished.

These closely related compounds are highly substituted coumarins, and the presence of the furocoumarin configuration places them among a large group of naturally occurring compounds with many pharmacological activities (63). The bifuran structure, however, has previously been encountered in only one other compound of natural origin, sterigmatocystin, a metabolite of *Aspergillus versicolor* (16). The importance of these structural configurations as determinants of

TABLE 2. *Summary of spectral data on aflatoxins*

Aflatoxin	Ultraviolet absorption (ε)		Infrared absorption (cm^{-1})					Fluorescence emission
	265 mμ	363 mμ	($\nu_{max}^{CHCl_3}$)					
								mμ
B_1	13,400	21,800	1,760	1,684	1,632	1,598	1,562	425
B_2	9,200	14,700	1,760	1,685	1,625	1,600		425
G_1	10,000	16,100	1,760	1,695	1,630	1,595		450
G_2	11,200	19,300	1,760	1,694	1,627	1,597		450

the biological activity shown by the aflatoxins remains to be established by further experimentation.

The spectral characteristics of the aflatoxins have been determined by several investigators (7, 8, 25, 33, 66, 67) and are summarized in Table 2. The ultraviolet absorption spectra are very similar, each showing maxima at 223, 265, and 363 mμ. The molar extinction coefficients at the latter two peaks, however, demonstrate that B_1 and G_2 absorb more intensely than G_1 and B_2 at these two wavelengths. Because of the close similarities in structural configuration, the infrared absorption spectra of the four compounds are also very similar, as illustrated. The fluorescence emission maximum for B_1 and B_2 has been reported to be 425 mμ, and that for G_1 and G_2 is 450 mμ (33). The intensity of light emission, however, varies greatly among the four compounds, a property of significance in the estimation of concentrations of the compounds by fluorescence techniques.

Chemical Properties

The chemical reactivity and behavior of the aflatoxins has received relatively little systematic study beyond work associated with structure elucidation. However, it has been shown (7, 8, 67) that catalytic hydrogenation of aflatoxin B_1 to completion results in the uptake of 3 moles of hydrogen with the production of the tetrahydrodeoxy derivative. Interruption of the hydrogenation procedure after the uptake of 1 mole of hydrogen results in the production of aflatoxin B_2 in quantitative yield (23, 67).

Aflatoxin B_1 has also been reported to react additively with a hydroxyl group under the catalytic influence of a strong acid (5). Treatment with formic acid-thionyl chloride, acetic acid-thionyl chloride, or trifluoroacetic acid results in addition products of greatly altered chromatographic properties, but relatively unchanged fluorescence characteristics. Ozonolysis results in fragmentation of aflatoxin B_1, and the products of this reaction include levulinic, succinic, malonic, and glutaric acids (67). The presence of the lactone ring makes the compound labile to alkaline hydrolysis, and partial recyclization after acidification of the hydrolysis product has been reported (25).

Although few systematic studies have been carried out on the stability of the aflatoxins, the general experience would seem to indicate that some degradation takes place under several conditions. The compounds appear partially to decompose, for example, upon standing in methanolic solution, and this process is greatly accelerated in the presence of light or heat. Substantial degradation also occurs on chromatograms exposed to air and ultraviolet or visible light. These processes may give rise to some of the non-aflatoxin fluorescent compounds typically seen in chromatograms of culture extracts. The nature of the decomposition products is still unknown, and the chemical reactions involved in their formation remain to be established.

BIOLOGICAL EFFECTS OF THE AFLATOXINS

The discovery of this group of compounds as contaminants of animal feeds, and the potential public health hazards involved, have stimulated considerable research effort concerned with their effects in various biological assay systems. The toxic properties of the aflatoxins manifest themselves differently depending on the test system, dose, and duration of exposure. Thus, they have been shown to be lethal to animals and animal cells in culture when administered acutely in sufficiently large doses, and to cause histological changes in animals when smaller doses were administered subacutely. Chronic exposure for extended periods has resulted in tumor induction in several animal species.

Acute Toxicity In Vivo

The aflatoxins are acutely toxic to most animal species. Aflatoxin B_1 has been most extensively studied, as regards its lethal potency, and its in vivo lethality to various experimental animals is summarized in Table 3. Early experimental studies, as well as observations in field cases in-

TABLE 3. *Comparative lethality of single doses of aflatoxin B_1*

Animal	Age (or weight)	Sex	Route*	LD_{50}	Reference
				mg/kg	
Duckling	1 day	M	PO	0.37	22
	1 day	M	PO	0.56	69
Rat	1 day	M-F	PO	1.0	69
	21 days	M	PO	5.5	69
	21 days	F	PO	7.4	69
	100 g	M	PO	7.2	18
	100 g	M	ip	6.0	18
	150 g	F	PO	17.9	18
Hamster	30 days	M	PO	10.2	
Guinea pig	Adult	M	ip	ca. 1	
Rabbit	Weanling	M-F	ip	ca. 0.5	
Dog	Adult	M-F	ip	ca. 1	
	Adult	M-F	PO	ca. 0.5	
Trout	100 g	M-F	PO	ca. 0.5	10

* PO = oral; ip = intraperitoneal.

volving contaminated feeds, suggested that the duckling was the species most susceptible to acute poisoning. The LD_{50} of -day-old ducklings is about 0.5 mg/kg. This value is considerably smaller than those for the rat and hamster, which are commonly used in toxicological evaluations. However, studies in progress in our laboratories indicate that the dog, rabbit, and guinea pig have LD_{50} values in the same order of magnitude as the duckling (Table 3). The same observation is true for the rainbow trout (10).

The LD_{50} values presented were calculated from mortality over 7-day periods. In most species, death usually occurred within the first 72 hr after administration of the compound, and necropsy at this stage generally revealed gross liver damage as the consistent pathological sign. Hemorrhage in the intestinal tract and peritoneal cavity and ascites were occasionally seen in some species. Butler (18) found the principal histological changes in rat liver to comprise the development of a periportal zone of necrosis over 3 to 4 days after dosing, with marked biliary proliferation. The latter lesions persist after 1 month.

In all species studied, sensitivity decreases with age, as illustrated by the rat data in Table 3 (1 day versus 21 days versus 100 g). The female rat is less susceptible to the acute effects, even at 21 days of age (weaning), and this sex difference appears to become more marked as sexual maturity is approached.

The relative lethal potencies of the four aflatoxins in the 1-day-old duckling have been examined by Carnaghan et al. (22). The oral 7-day LD_{50} values reported for each compound were: aflatoxin B_1, 18.2 μg; B_2, 84.8 μg; G_1, 39.2 μg; and G_2, 172.5 μg, all values in this instance being reported on a 50-g body weight basis. These values illustrate clearly the relationships of structural configuration to acute lethality. Aflatoxin B_1 is most potent, followed by G_1, B_2, and G_2 in order of decreasing potency. The presence of the additional oxygen in the G compounds results in activity decreased by a factor of about 2, whereas the unsaturated compounds are approximately 4.5 times as potent as the dihydro derivatives.

Acute Toxicity in Cell Cultures and Embryos

The toxic effects of aflatoxin B_1 have also been investigated in several in vitro cell culture systems and in embryonated eggs. These experimental systems are much more sensitive than in vivo systems in terms of the amounts of toxin required to produce effects. The results of several such studies are shown in Table 4. The early studies of Juhasz and Greczi (37) demonstrated that methanol extracts of peanut meals containing aflatoxins are toxic to calf kidney cells. No accurate estimation of the aflatoxin concentrations was available. Legator and Withrow (41) have shown that very small concentrations of aflatoxin B_1 inhibit the mitotic process in human embryonic lung cells. Gabliks et al. (29*a*) reported that concentrations of the compound in the range of 1 to 5 μg/ml of medium cause destruction (TD_{50}) of human liver and HeLa cells as well as primary cell cultures of whole duck and chick embryos. Small concentrations of toxin caused demonstrable effects such as inhibition of protein synthesis. It is interesting to note the apparently greater sensitivity of duckling embryo cells as compared with that of chick embryos, since this finding agrees with relative in vivo susceptibility.

Platt et al. (51) observed that aflatoxin mixtures are toxic to chick embryos, and this observation has been amply confirmed and extended with aflatoxin B_1. The data of Verrett et al. (68) indicate the sensitivity of the chick embryo, in which the LD_{50} is 0.048 μg/egg when administration is made via the yolk, and 0.025 μg/egg when the compound is applied in the air cell. In both cases, the fertilized eggs were treated prior to incubation. The data of Gabliks et al. (29*a*) indicate somewhat reduced sensitivity in older embryos receiving the toxin via the chorioallantoic cavity, but again suggest that the duckling embryo is more susceptible than the chick. Embryo toxicity therefore provides a useful tool by virtue of the small quantity of compound required to produce an effect. This level of sensitivity makes possible the use of the embryo response as a means of evaluating the biological activity of aflatoxin metabolites, derivatives, and degradation products which are presently obtainable in only small

TABLE 4. *Toxicity of aflatoxin B_1 in cell cultures and embryos*

Cell cultures

Cells	Aflatoxin concn	Effects	Reference
	μg/ml		
Calf kidney	?	Nuclear and cytoplasmic destruction in 48 hr	37
Human embryonic lung	0.03	Reduction in mitotic rate (51% in 8 to 12 hr)	41
Human (Chang) liver	1.0	TD_{50} in 48 hr	
HeLa cells	5.0	TD_{50} in 48 hr	
Primary duck embryo	1.0	TD_{50} in 48 hr	
Primary chick embryo	5.0	TD_{50} in 48 hr	

Embryonated eggs

Cells	LD_{50}	Route	Reference
	μg/egg		
Chick (5-day)	0.3	Yolk (?)	51
Chick (preincubation)	0.048	Yolk	68
	0.025	Air cell	
Chick (10-day)	2.0–5.0	Chorioallantoic cavity	
Duck (15-day)	0.5–1.0	Chorioallantoic cavity	

amounts. The relative lack of specificity of the embryo lethality response, however, requires that the test materials be available in pure form.

Subacute Toxicity

The toxicity of the aflatoxins was discovered as a result of accidental poisoning of several domestic animal species, as described earlier. Although these incidents involved relatively uncontrolled observations, and few experiments have been carried out in these animals, several observations from the field cases have provided useful information. In all species, animals which consumed sublethal quantities of the compounds for several days or weeks developed a subacute toxicity syndrome which commonly included moderate to severe liver damage as a prominent pathological sign. Among the various domestic species, several types of histopathological lesions of the liver have been described.

The nature of these lesions and the variation in their appearance in several species are summarized in Table 5. Consideration of these data permits two pertinent conclusions. First, the sheep appears to be unique among the species studied in its resistance to the toxin, an observation which has been verified experimentally (2). Also, the development of biliary hyperplasia represents the most consistently observed lesion, occurring in all species except the sheep.

The extent and severity of this histopathological change can be evaluated in semiquantitative terms by the pathologist, and has been used as a criterion of aflatoxin activity, particularly in the young duckling (69). This response provided the basis for biological assays for aflatoxin contamination of foodstuffs (6, 49), and the response has been studied in some detail (17, 48, 71). When the toxin is administered repeatedly to the 1-day-old duckling over a 5-day period, characteristic lesions are observed on the 7th day. Detailed examination (69) of the quantitative aspects of this response has revealed that the administration of aflatoxin B_1 to ducklings according to this dose schedule results in reproducible responses at a dose level of 0.5 μg per day (approximately 2.5 μg, total dose). This level of sensitivity has made the assay useful in detection of contamination of foodstuffs.

Subacute toxic effects of the aflatoxins in monkeys have been reported by Tulpule et al. (65). In these experiments, young (1.5 to 2.0 kg) rhesus monkeys were fed either 0.5 or 1.0 mg of aflatoxin per day for 18 days, then 1.0 mg/day thereafter. All animals developed anorexia and died in 14 to 28 days. The principal histopathological findings included liver lesions similar to those seen in ducklings (portal inflammation and fatty change), suggestive of biliary cirrhosis. In

TABLE 5. *Comparative pathology in animals fed aflatoxin-contaminated feed*

Liver lesions	Calves	Cattle	Swine	Sheep	Duckling	Adult duck	Turkey poult	Chick
Acute necrosis and hemorrhage	−	−	+	−	+	−	+	−
Chronic fibrosis	+	+	+	0	−	+	−	−
Regeneration nodules	−	+	+	0	±	+	+	−
Bile duct hyperplasia	+	+	+	0	+	+	+	±
Veno-occlusive disease	+	+	−	0	−	−	−	−
Enlarged hepatic cells	+	+	+	0	+	+	+	−
Liver tumors	0	0	0	0	−	+	0	0

these experiments, the animals received a total dose of 10 to 15 mg/kg of body weight of a preparation containing 60% aflatoxin B_1 and 40% G_1. This report comprises the only published information available to date concerning the effects of the compounds in primates.

Carcinogenic Properties

The acute potency of the aflatoxins is clearly established by the effects described in the preceding section. Equally interesting and significant effects result from prolonged administration of sublethal quantities of the compounds to animals. In early investigations of aflatoxin-contaminated peanut meals, investigators at the Unilever Research Laboratories in England (39) fed diets containing highly toxic peanut meals to rats. After 6-months feeding of 20% peanut meal (aflatoxin content not known) in a purified diet, 9 of 11 rats developed multiple liver tumors, and two of these developed lung metastases. This finding represented the first indication of the carcinogenic properties of aflatoxin-contaminated, toxic peanut meals, and has since been amply confirmed (12, 20, 40, 53, 57). Subsequent investigations have been concerned with the demonstration that the aflatoxins were the responsible carcinogenic agents and with the determination of dose, duration of exposure, and other conditions for tumor induction by these compounds.

Although precise dose-response conditions have not yet been established, some information is available regarding the dose-response relationships between tumor incidence in rats and aflatoxin content of contaminated peanut meals. The results of studies on several such meals have been described by Newberne (47), who reported good correlation between liver tumor incidence and dietary aflatoxin content over the range of 0.06 to 1.8 ppm of aflatoxin. The highest level resulted in more than 90% tumor incidence when fed over a period of 370 days. The lowest level of aflatoxin detected (0.005 ppm) failed to induce liver tumors within a similar time period (384 days).

Mice appear to be relatively resistant to acute poisoning by the aflatoxins, as evidenced by results of short-term feeding of heavily contaminated peanut meals. Platonow (50) studied effects of diets containing 15, 30, or 80% of a peanut meal containing 4.5 ppm each of aflatoxin B_1 and G_1 and 0.6 ppm each of aflatoxins B_2 and G_2. These diets were fed to groups of mice (20 to 25 g) for at least 3 months. During this time, no effects were noted on feed intake or body weight, and no deaths occurred. Histopathological examination revealed no significant changes in liver or other tissues studied.

TABLE 6. *Rat hepatoma incidence after administration of purified aflatoxins*

Aflatoxin	Feeding time	Tumor incidence
	days	
1.75 ppm*	89	1/3 (316 days later) 2/2 (485 days later)
150 μg/day	30	3/3 (5 months later) 2/2 (10 months later)
75 μg/day	30	4/5 (10 months later)
37.5 μg/day	30	5/5 precancerous lesions (10 months later)
15 μg/day	30	4/5 precancerous lesions (10 months later)

* In diet; data of Barnes and Butler (12).

Newberne (47) reported that, in a group of mice fed diets containing aflatoxin-contaminated peanut meals for a period of 16 months, 6 of 40 survivors developed hepatomas which were similar to those observed in ducklings. However, the significance of this observation is not certain, since the mouse tumors did not appear to be histologically malignant and metastatic lesions were not observed.

Recent experiments with purified aflatoxin preparations have indicated that continuous feeding is not required for hepatoma induction in rats. The results of two such investigations are summarized in Table 6. In the studies of Barnes and Butler (12), rats were fed 1.75 ppm of aflatoxin (containing 80% aflatoxins, with G_1 present in higher quantities than B_1) in the diet for 89 days, and were then returned to an aflatoxin-free diet. All of three treated animals ultimately developed liver cancer after more than 300 days following withdrawal.

In somewhat similar experiments in our laboratories (Table 6), we administered an unfractionated mixture of partially purified aflatoxins (approximately 30% B_1, 20% G_1) to rats by stomach tube. Each animal was treated daily for 30 days, and then was held without treatment for a further 10 months. Animals which received the highest dose (150 μg/day) had well-developed liver tumors 5 months after withdrawal of treatment. Even those rats receiving the lowest dose studied (15 μg/day) showed significant incidence of precancerous lesions at the same time interval. These lesions probably would have progressed to tumors over a longer period of time. These data would indicate that continuous exposure to the compounds is not required for liver tumor induction.

On the basis of these preliminary data, it has been possible to estimate the effective dose of aflatoxin B_1 for the induction of liver tumors in rats. Butler (19) estimated this dose to be in the order of 10 μg per day. When this value is compared with similar estimates for other hepatocarcinogens, such as dimethylnitrosamine (750 μg/day) and butter yellow (9,000 μg/day), the relative potency of the compound is readily apparent.

Recent studies by Ashley et al. (9, 10) and by Sinnhuber et al. (61) have suggested that the rainbow trout may be considerably more sensitive than the rat to the hepatocarcinogenic effects of the aflatoxins. These investigators have shown that rainbow trout develop liver tumors at significant incidence rates when fed purified diets containing only 0.5 to 2.0 μg of aflatoxin B_1 per kilogram (i.e., 0.5 to 2.0 ppb). The apparent sensitivity of this species had led to the recognition (10) of the potential role of the aflatoxins as etiological agents in the so-called "trout hepatoma syndrome" (31, 36).

In a different test system, Dickens and Jones (28) studied the effects of multiple subcutaneous injections of aflatoxins B_1 and G_1 in rats. A mixed preparation (about 38% B_1 and 56% G_1) of the compounds dissolved in peanut oil was administered to groups of rats twice weekly. One group received 50 μg at each injection, and the treatment was continued for 50 weeks; a second group received 500 μg at each injection for a period of only 8 weeks, after which treatment was discontinued. In the former group, six of six animals developed sarcomas or fibrosarcomas at the injection site within a 60-week period. At the higher dose level, five of five animals developed tumors within a 30-week period. These observations indicate that the compounds are also carcinogenic for the subcutaneous tissues of the rat. Surprisingly, only one of the aflatoxin-treated animals at the higher dose level showed moderate liver lesions comparable to those resulting from oral administration.

METABOLISM OF AFLATOXINS BY ANIMALS

The metabolic fate of the aflatoxins in animals is of interest in connection with studies related to the mode of action of the compounds. Although available information is not yet extensive, some aspects of the problem have been approached in several investigations.

De Iongh et al. (27) observed that lactating cattle fed subtoxic levels of aflatoxin-containing peanut meals excreted in their milk a compound which was toxic to ducklings. Although the lesions caused by the so-called "milk-toxin" were similar to those caused by aflatoxin B_1, the toxicity was associated with a violet-fluorescent compound with greatly altered chromatographic properties. Little, if any, aflatoxin B_1 was detected in extracts of toxic milk, indicating that the active compound may represent a metabolite. Subsequent experiments demonstrated that lactating rats also convert aflatoxin B_1 into the toxic metabolite.

TABLE 7. *Distribution of C^{14} from radioactive aflatoxin B_1 in male rats**

Distribution	Methoxy-labeled aflatoxin B_1		Ring-labeled aflatoxin B_1	
	Rat 1	Rat 2	Rat 3	Rat 4
Total excreted	68.2	72.8	78.0	84.9
Expired CO_2	20.6	32.6	0.3	0.3
Urine	13.8	26.1	20.6	14.8
Feces	33.8	14.1	57.1	69.8
Liver	9.2	5.9	9.9	7.7
Stomach and contents	2.6	0.3	<0.1	0.3
Intestines and contents	6.8	11.8	4.7	3.3
Blood	8.0	0.8	1.9	2.3
Kidneys	0.2	0.4	0.5	0.4
Muscle	<0.1	<0.1	<0.1	<0.1
Brain	<0.1	<0.1	0.0	0.0
Weight of rat (g)	122	40	62	56
Dose (μg)	337	165	6	7
Dose (counts per min $\times 10^6$)	4.18	2.07	1.65	1.98
Total C^{14} recovered (% of dose)	107.6	105.8	97.5	97.5

* Results are expressed as the percentage of C^{14} recovered 24 hr after a single intraperitoneal dose.

In our laboratories, studies concerning the metabolic fate of aflatoxin B_1 in rats have been facilitated by the availability of C^{14}-labeled compounds produced in the course of biosynthesis investigations by Adye and Mateles (1). Two types of labeled compounds have been prepared. In one instance, aflatoxin B_1 labeled in the methoxy carbon (Fig. 1) has been obtained by addition of methyl-labeled methionine to the culture medium. The C^{14} appears in aflatoxin B_1 only in the methoxy function. Addition to the medium of acetate-*1*-C^{14} as the sole carbon source yields aflatoxin labeled only in the ring carbons. Although this compound is presumably uniformly labeled in the ring positions, this distribution has not been experimentally verified.

We have studied the distribution and excretion of C^{14} during the 24-hr period following intraperitoneal administration of the labeled compounds to rats (60). Results of four typical experiments are summarized in Table 7. When the methoxy-labeled compound was used, in the order of 25 to 30% of C^{14} was recovered in CO_2. An additional 25% was excreted in urine; feces and intestinal contents accounted for approximately 25% of the recovered dose. The liver,

which contained 6 to 9% of the dose after 24 hr, was the only tissue that retained significant amounts of radioactivity.

When ring-labeled compound was administered, the pattern of distribution was somewhat altered. In this case, virtually no activity appeared in CO_2, but was largely excreted in urine and feces. In these animals, the liver also retained approximately 8% of the radioactivity at the end of the study period.

It is noted that 70% of the C^{14} from the methoxy-labeled aflatoxin is excreted, with about 25% in CO_2. This indicates that O-demethylation represents one substantial aspect of the metabolism of the compound, a fact which makes the compound labeled in this position of limited usefulness in studies of its distribution in animals. Slightly more of the ring-labeled compound was excreted, with practically none of the radioactivity appearing in CO_2. These results suggest that the ring structures are metabolically stable. The large amounts of radioactivity found in feces and intestinal contents are thought to indicate excretion of major quantities of the compound via the bile. This suggestion is supported by the findings of Falk et al. (29), who reported rapid appearance of aflatoxin and a series of fluorescent metabolites in bile after intravenous injection of aflatoxin into rats.

The chemical nature of the excreted metabolites and of the residual compounds has not yet been determined. Little aflatoxin appears to be excreted unchanged, but rather appears in urine and feces in forms with greatly altered solubility and chromatographic properties. The relationships of the metabolic fate of the compound to the kinetics and mechanism of its action in causing acute liver damage or in the induction of liver tumors await further experimentation.

Biochemical Effects of Aflatoxins

Biochemical alterations caused by aflatoxins have been studied in a number of biological systems. Smith (62) investigated the effects of aflatoxin B_1 on the rate of in vitro incorporation of leucine-C^{14} into proteins by rat and duckling liver slices. Significant reduction in the rate of incorporation in 80-mg rat liver slices was caused by addition of 10 μg of toxin, and almost complete suppression was effected by 200 μg. Duckling liver slices studied under similar conditions were more sensitive, showing significant reduction with 3.5 μg of toxin. These observations suggested that the compound suppresses protein synthesis by some mechanism, and this activity has been investigated in other test systems.

We have studied (59) the in vivo incorporation of leucine into rat liver proteins after single sublethal doses of aflatoxin B_1. A biphasic response in incorporation was observed after a single oral dose of 4.76 mg of toxin/kg of body weight, in which the toxin immediately suppressed, then increased, amino acid incorporation. Inhibition was clearly evident within 30 min after dosing, and was maximal at 6 hr, at which time the rate of incorporation was reduced to approximately 50% of the control value. This was followed by a 72-hr period during which the incorporation in the treated animals exceeded the control rate, being maximal 72 hr after toxin administration.

These results led to further investigation of effects on synthesis of specific proteins. For this purpose, we determined the influence of the toxin on liver tryptophan pyrrolase induction by hydrocortisone and by tryptophan in rats (70). The increase in activity of this enzyme in liver 6 hr after intraperitoneal injection of 150 mg of hydrocortisone/kg, or 600 mg of tryptophan/kg, was studied in weanling male rats. Animals receiving aflatoxin B_1 at a level of 1 mg/kg and hydrocortisone showed no increase in enzyme activity 6 and 12 hr after toxin administration, as compared to a fourfold increase in those receiving hydrocortisone only. Although this inhibitory effect was not apparent 5 days later, larger doses (3 or 5 mg/kg) of aflatoxin caused inhibition of enzyme induction which persisted for at least 10 days. Animals treated with 5 mg of toxin/kg showed no significant inhibition of enzyme induction by tryptophan. The inhibition of hydrocortisone induction, which involves de novo enzyme synthesis, and the failure to inhibit tryptophan induction, which depends upon enzyme stabilization without synthesis, suggests that the toxin is an inhibitor of protein synthesis at some specific stage. The locus of this effect is currently under investigation.

The aflatoxins also have interesting effects in tissues of plant origin. Schoental and White (58) showed that aflatoxins in concentrations of 25 μg/ml inhibit the germination of the seeds of cress (*Lepidium sativum* L.). Smaller concentrations apparently interfered with chlorophyll synthesis, since there was complete absence of color when the germinating seeds were exposed to the compounds in concentrations of 10 μg/ml.

Black and Altschul (13) recently reported the finding that gibberellic acid-induced increases in lipase and α-amylase activity of the germinating cottonseed are inhibited by aflatoxin. This effect is similar qualitatively to the inhibition of enzyme induction in rat liver described above, although it is not yet clear whether the mechanism of inhibition is related in the two systems.

SUMMARY

The information reviewed here emphasizes the importance of the discovery of the aflatoxins. This discovery, arising from astute observations in a seemingly obscure toxicity syndrome in poultry flocks, has led to increasing general attention to the possible significance of toxic mold metabolites as contaminants of foodstuffs and therefore as potential etiological agents in problems of animal and human health. In addition, the potency of these compounds as toxic agents and as carcinogens should make them useful and powerful tools in investigations into the mechanism of toxicity and chemical carcinogenesis.

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